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Original Article

Effect of umbilical cord blood stem cells transplantation on bladder dysfunction induced by cerebral ischemia in rats

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ABSTRACT

Objective: To demonstrate the effect of human umbilical cord blood-derived $CD34^+$ cells on bladder dysfunction induced by cerebral ischemia in rats.
Materials and Methods: Female rats were subjected to either 60 minute middle cerebral artery occlusion (MCAO) or a sham operation. Rats were divided into four groups: sham operation, MCAO without treatment, infusion with 1×10^6 $CD34^+$ cells 30 minutes before MCAO, and infusion with 1×10^6 $CD34^+$ cells 3 hours after MCAO. Bladder function was analyzed by cystometry at 1 day, 3 days, and 7 days after MCAO. Expressions of nerve growth factor (NGF), M_2 and M_3 muscarinic receptors were measured by immunohistochemistry and real time polymerase chain reaction.
Results: Cystometric results showed that, following MCAO, rats have a significant increase in peak voiding pressure and residual volume. Conversely, there is a significant decrease in voided volumes and intercontraction intervals. Cystometric variables after pre- and postischemic $CD34^+$ treatment nearly returned to levels found in sham-operated rats. The expression of bladder NGF and M_3 was decreased after MCAO, but significantly increased following preischemic $CD34^+$ treatment. There was decreased expression of bladder M_2 mRNA despite an increased level of M_2 immunoreactivity at 3 days and 7 days after MCAO. Expression of bladder M_2 immunoreactivity and mRNA nearly returned to sham group levels after preischemic $CD34^+$ treatment.
Conclusion: Bladder dysfunction in a rat model caused by MCAO may be restored to normal micturition by treatment with human umbilical cord blood-derived $CD34^+$ cells and may be related to the expressions of NGF, M_2, and M_3 in the bladder.

Introduction

Cerebral stroke has ranked as one of the leading causes of death worldwide. Poststroke mortality and chronic neurological disability are the result of neuronal damage for which there is currently no effective preventive treatment. Recent data suggest that human umbilical cord blood (HUCB)-derived $CD34^+$ cells may promote functional recovery in an animal model of stroke [1]. Several mechanisms of HUCB recovery action were suggested including reduction of damaged cells, recognization of nerve fibers by trophic actions, reduction in inflammatory response, and enhancement of angiogenesis [1]. However, no study has been conducted to demonstrate that HUCB-derived $CD34^+$ cells can mediate therapeutic effects on bladder dysfunction following cerebral stroke, although voiding dysfunction is common in patients with cerebrovascular diseases.

Bladder overactivity induced by middle cerebral artery occlusion (MCAO) in rats has been found to involve receptors in the brain such as dopamine, glutamate, and gamma-aminobutyric acid [2–6]. Nerve growth factor (NGF) and muscarinic receptors in cerebral-infarcted rats also play a role in regulation of the micturition reflex in central and peripheral nervous systems [4,7,8]. There is a significant reduction of brain NGF after severe cerebral ischemia

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[7]. NGF has an effect on bladder dysfunction by mediating morphological and functional changes in sensory neurons innervating the bladder [8]. Muscarinic receptors in the brain contribute to control of the micturition reflex [4]. A significant increase in the density of muscarinic receptors has been found in the urinary bladder of cerebral-infarcted rats [9]. The present study was conducted to evaluate whether: (1) the protective effect of HUCB-derived $CD34^+$ cells against cerebral vascular disease may improve bladder dysfunction; and (2) HUCB-derived $CD34^+$ cells treatment may sustain the expression of NGF and muscarinic receptors in the bladder.

Materials and methods

Animal model

All protocols were approved by the Institutional Ethics Committee for the Care and Use of Experimental Animals and the Institutional Review Board of Chang Gung Memorial Hospital Linkou Medical Center, Taoyuan, Taiwan. Female Sprague Dawley rats from the National Laboratory Animal Center (Taipei, Taiwan) were maintained at 21–23°C room temperatures and 47% humidity with a 12-hour light-dark cycle with free access to standard laboratory chow and tap water. Because endogenous estrogen may improve stroke outcome during MCAO [10], all rats in this study underwent bilateral ovariectomy performed 2 weeks before MCAO to create a hypoestrogenic menopausal state. Rats were subjected to either 60 minute MCAO or sham operation. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Number 80–23) revised 1996.

Clinically available tools such as recombinant tissue plasminogen activator were found to have a therapeutic window of 3–5 hours for the treatment of stroke [11]. In order to study the effect of HUCB-derived $CD34^+$ cells on the bladder dysfunction before and after ischemic insult, 72 rats (270–320 g) were randomly assigned to four groups: (1) sham operation; (2) rats with MCAO but no $CD34^+$ cells treatment; (3) rats infused with 1×10^6 $CD34^+$ cells 30 minutes before MCAO (preischemic treatment group); and (4) rats infused with 1×10^6 $CD34^+$ cells 3 hours after MCAO (postischemic treatment group). Bladder function was analyzed using conscious cystometry at 1 day, 3 days, and 7 days after MCAO (6 rats in each time point). Expressions of NGF, M_2 muscarinic receptor (M_2), and M_3 muscarinic receptor (M_3) were measured by immunohistochemistry and real time polymerase chain reaction (PCR).

Induction of cerebral ischemia

Left MCAO was used as an acute ischemic model according to previously reported methods [12]. Rats were anesthetized with 2% isoflurane in oxygen. Following satisfactory anesthesia, the proximal portion of the external carotid artery was tightly ligated with a silk suture. A 20 mm 4-0 nylon surgical thread was inserted from the left external carotid artery into the internal carotid artery to occlude the MCA. The left common carotid artery was then permanently ligated and the wound was temporarily closed.

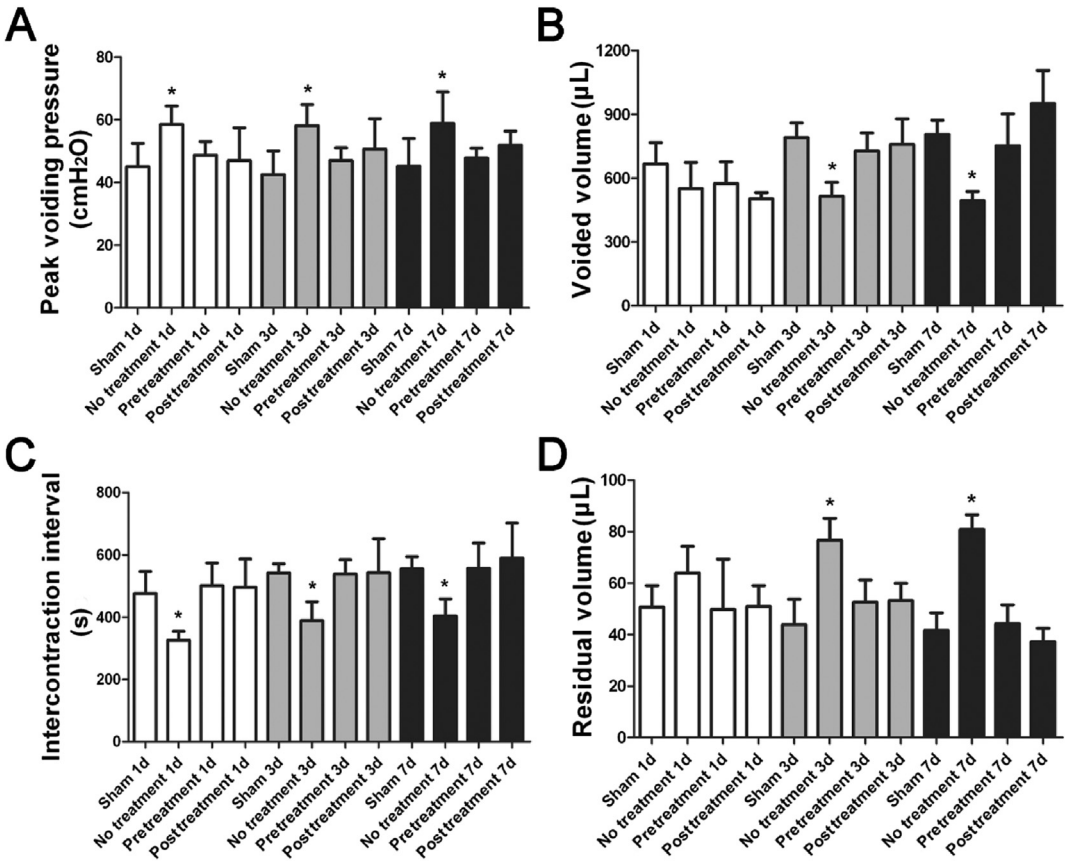

Figure 1. Cystometric results in the experimental rats (A–D) are presented (6 rats in each group). Ischemic rats show a significant increase in peak voiding pressure (A) and residual volume (D) but significant decrease in voided volume (B) and intercontraction interval (C) from 1 day to 7 days after middle cerebral artery occlusion (MCAO). *Compared with sham-operated group, $p < 0.05$. Pretreatment: preischemic treatment, posttreatment: postischemic treatment.

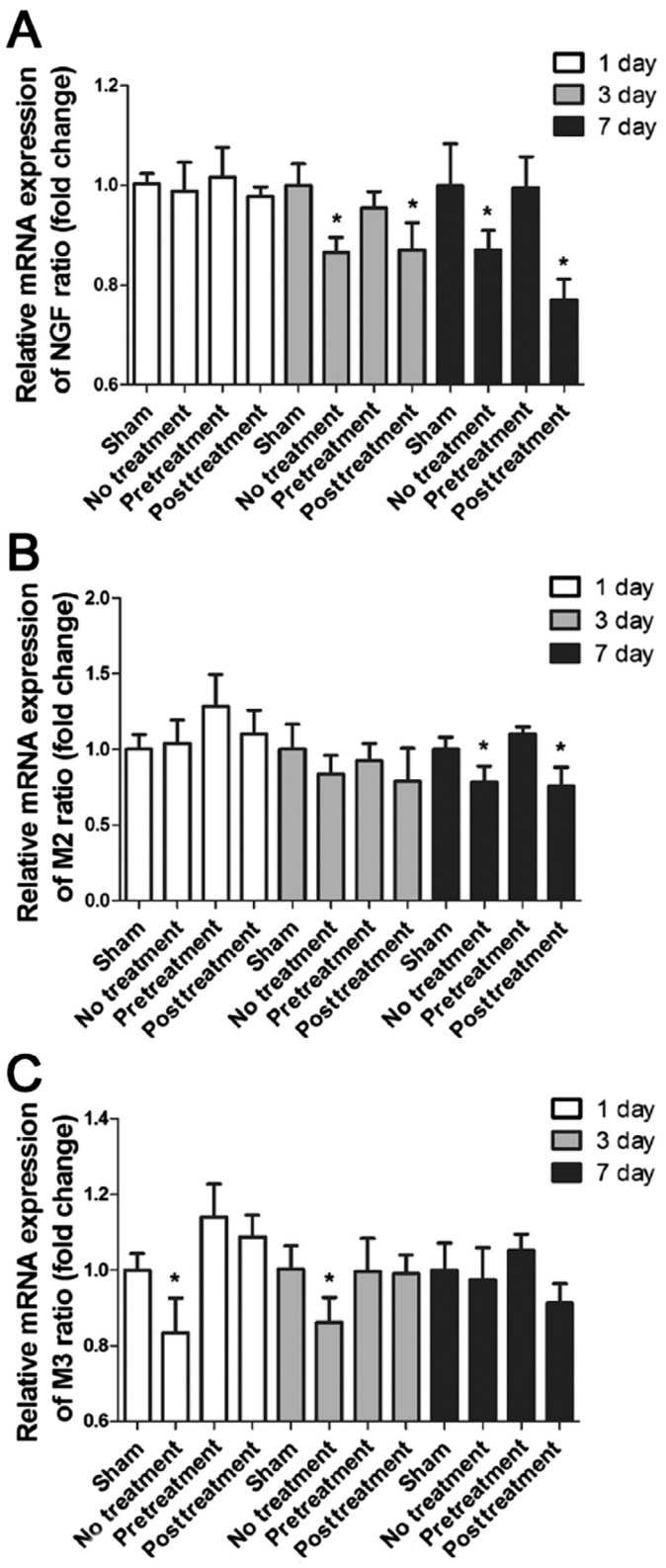

Figure 2. Relative mRNA expression ratio of (A) nerve growth factor (NGF), (B) M_2, and (C) M_3 (C) after middle cerebral artery occlusion (MCAO). The mRNA expressions of NGF and M_2 decrease after MCAO but increase at 7 days following preischemic $CD34^+$ cell treatment (6 rats in each group). M_3 mRNA expression significantly decreases after MCAO but increases at 1 day and 3 days after $CD34^+$ cell treatment. *Compared with sham-operated group, $p < 0.05$. Pretreatment: preischemic treatment, posttreatment: postischemic treatment.

Anesthesia was discontinued after these procedures were complete. After a 60-minute occlusion of the left MCA, rats were reanesthetized, and the wound was opened to remove the nylon surgical thread to allow for the reperfusion of the MCA. In the sham-operated group, similar procedures were conducted without ligation or occlusion of any vessel. Endovascular suture occlusion of the MCA for 60 minutes would result in irreversible cerebral ischemic injury in both the cerebral cortex and striatum [12]. Therefore, we only included animals that exhibited right side weakness with upper limb dominance.

Preparation of $CD34^+$ cells for transplantation

After informed consent, HUCB was obtained from healthy individuals using a VarioMACS Starting Kit (Miltenyi Biotec, Bergisteh Gladbach, Germany) and CD34 MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol. The $CD34^+$ cells were collected and washed with phosphate-buffered saline (PBS) and then prepared to a final concentration of 1×10^6 cells/0.3 mL in PBS [13]. In the $CD34^+$-treated groups, 1×10^6 collected $CD34^+$ cells were administered 30 minutes before or 3 hours after MCAO via the tail vein under inhalation anesthesia.

Suprapubic tube implantation and cystometric studies

All rats received suprapubic tube implantation under isoflurane general anesthesia 5 days prior to implementation of MCAO. The animals were later placed in special metabolic cages (Med Associates, St. Albans, VT, USA) after MCAO to perform conscious cystometric studies according to methods described in our previous study [14]. Briefly, the suprapubic catheter was connected to a syringe pump and pressure transducer. All bladder pressures were referenced to air pressure at the level of the bladder. Pressure and force transducer signals were amplified, recorded on a chart recorder and digitized for data collection. The bladder was then filled with room temperature 0.9% saline at 5 mL/h through the bladder catheter while bladder pressure was recorded. Urine was collected in a beaker on a balance placed beneath each cage. Changes in the weight of the collection were recorded. Saline infusion was continued until rhythmic bladder micturition contractions became stable. After the initial stabilization period, data for five representative micturition cycles were collected to analyze all cystometric parameters. The following cystometric variables were investigated: peak voiding pressure, intercontraction interval, voided volume, and residual volume. Cystometry Analysis version 1.05 (Catamount Research and Development, St. Albans, Vermont, USA) was used for cystometric analysis.

Immunohistochemistry

After cystometric studies were completed, the rats were sacrificed and their bladders were harvested. The dissected bladders were fixed in an optimal cutting temperature compound, frozen in powdered dry ice, and stored at −80°C. The bladders were then subjected to cryosectioning (10 μm) at −18°C with the sections mounted on glass microscope slides coated with saline (Muto Pure Chemical, Tokyo, Japan).

Immunostaining against NGF, M_2, and M_3 in fresh-frozen bladder sections was performed with an avidin-biotin peroxidase method using a kit (PK-6102, Vector Laboratories, Burlingame, CA, USA). First, fresh-frozen sections were fixed in 4% paraformaldehyde for 10 minutes, air dried, and then rinsed with PBS. After blocking with Dako REAL peroxidase blocking solution (code S2023, DAKO Corporation, Carpinteria, CA, USA) for 20 minutes, sections were washed and incubated for 18–20 hours at 4°C with a

rabbit polyclonal antibody directed against NGF at a 1:250 dilution (Chemicon International, Temecula, CA, USA) or rabbit polyclonal antibody directed against M_2 and M_3 at a 1:2000 dilution (Chemicon International). Endogenous peroxidase was blocked for 10 minutes with 0.2% H_2O_2 and 100% methanol. Then, sections were washed and incubated for 1 hour using biotinylated anti-rabbit immunoglobulin G antibody for NGF, M_2, and M_3 at a 1:500 dilution (Vector Laboratories). This was followed by incubation for 1 hour with avidin-biotin-horseradish peroxidase complex at a 1:100 dilution. Staining was developed with 3,3′-diaminobenzidine plus hydrogen peroxide as the chromogen. All incubations were performed at room temperature except for the use of primary antibody. Negative control slides were prepared from the same tissue blocks by omitting the specific primary antibodies and using normal, nonimmune serum supernatant from the same sources. The ratio of the optical density of no treatment, pre-, or post-treatment MCAO rat to that of sham-operated rat was determined in NGF, M_2, and M_3 analyses. Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA) was used for immunohistochemical measurement calculations.

Real-time PCR

Real time PCR was carried out according to the manufacturer's protocol. Total RNAs were prepared using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and incubated in reverse transcription mixture at 25°C for 5 minutes, 50°C for 1 hour, and 70°C for 15 minutes; finally, the tubes were cooled to 4°C for 5 minutes. Gene expression for NGF, M_2, and M_3 in the bladder tissue was analyzed by real time PCR using inventoried TaqMan assays from Applied Biosystems (Life Technologies, Grand Island, NY, USA). The NGF, M_2, and M_3 assays codes were Rn01533872-m1, Rn02532311-s1, and Rn00560986-s1, respectively (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) assays codes (Rs99999916-s1) were used as an endogenous control to allow for quantification of relative gene expression. Thermal cycling and fluorescence detection were performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

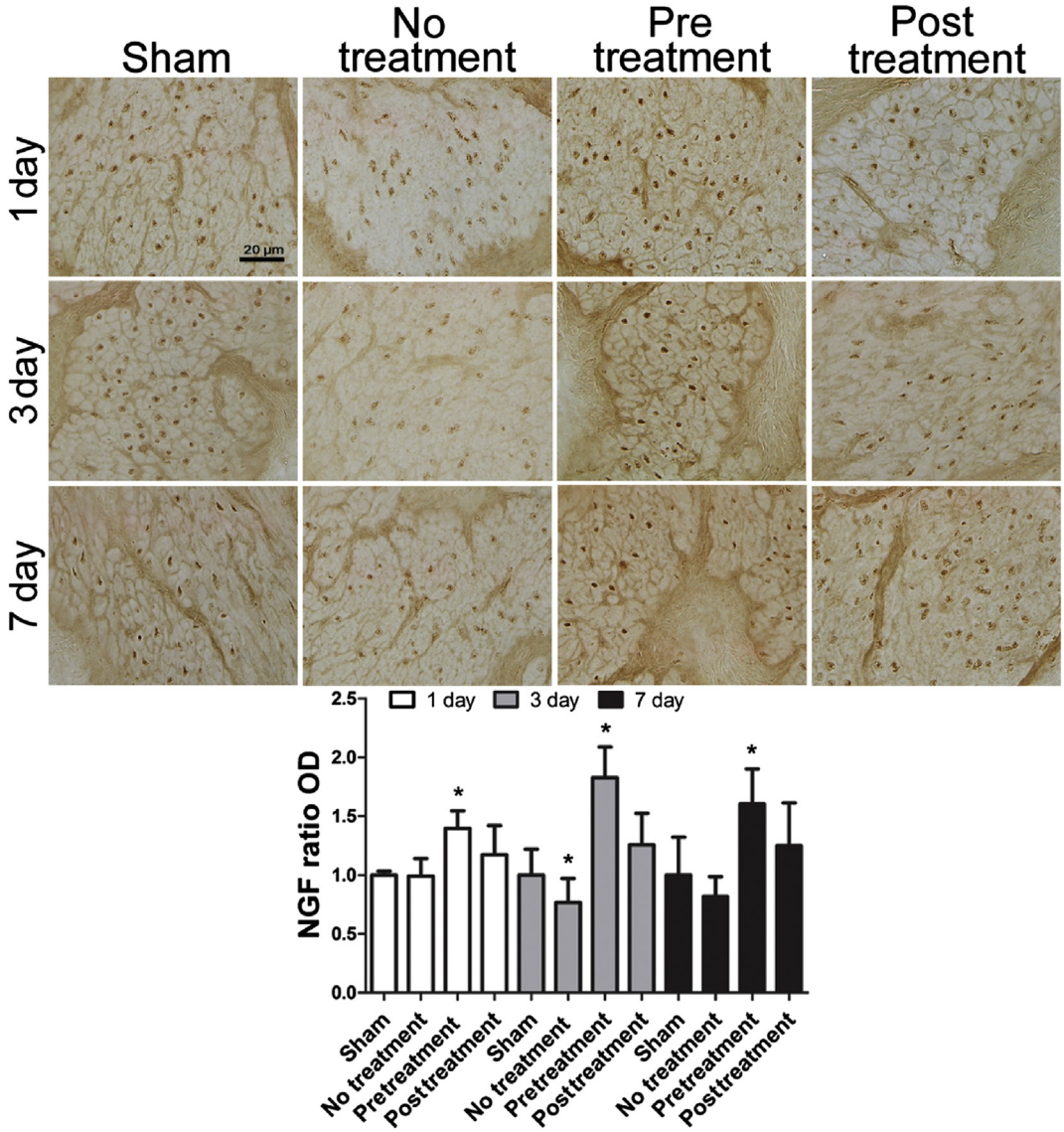

Figure 3. Temporal expressions of nerve growth factor (NGF) immunoreactivity in middle cerebral artery occlusion (MCAO) rat bladder (6 rats in each group). Expression of NGF immunoreactivity decreases at 3 days and 7 days after MCAO, but significantly increases following preischemic $CD34^+$ cell treatment. Bar indicates 20 μm. *Compared with sham-operated group, $p < 0.05$. Pretreatment: preischemic treatment, posttreatment: postischemic treatment.

The data were calculated using the 2[-Delta Delta C(T)] method [15]. A ratio of the mRNA level of ischemic rats to that of sham-operated rats was determined. The values were summed and expressed as mean ± standard deviation and were compared statistically among sham operation and each time point in each group.

Statistical analysis

The data were analyzed statistically using one-way analysis of variance test followed by a Tukey test. Values were considered significant at $p < 0.05$. Prism 5 software for statistical analysis (GraphPad, San Diego, CA, USA) was used for all data.

Results

Cystometric results showed ischemic rats have an increase in peak voiding pressure and residual volume at 1 day, 3 days, and 7 days following MCAO, which declined after pre- and postischemic $CD34^+$ cells treatment (Figure 1). Voided volumes and intercontraction intervals significantly decreased after MCAO, but increased after pre- and postischemic $CD34^+$ cells treatment at 3 days and 7 days.

The mRNA expressions of NGF and M_2 decreased after MCAO, but increased at 7 days following preischemic $CD34^+$ cell treatment (Figure 2). M_3 mRNA expression significantly decreased after MCAO, but increased at 1 day and 3 days after $CD34^+$ cell treatment.

Expression of NGF immunoreactivity decreased at 3 days and 7 days after MCAO, but significantly increased following preischemic $CD34^+$ cell treatment (Figure 3). Expression of M_2 immunoreactivity increased at 3 days and 7 days after MCAO, but declined to the levels of the sham-operated group after $CD34^+$ treatment (Figure 4). Expression of M_3 immunoreactivity significantly decreased at 1 day, 3 days, and 7 days after MCAO, but increased after $CD34^+$ cell treatment (Figure 5). Unlike the effect of

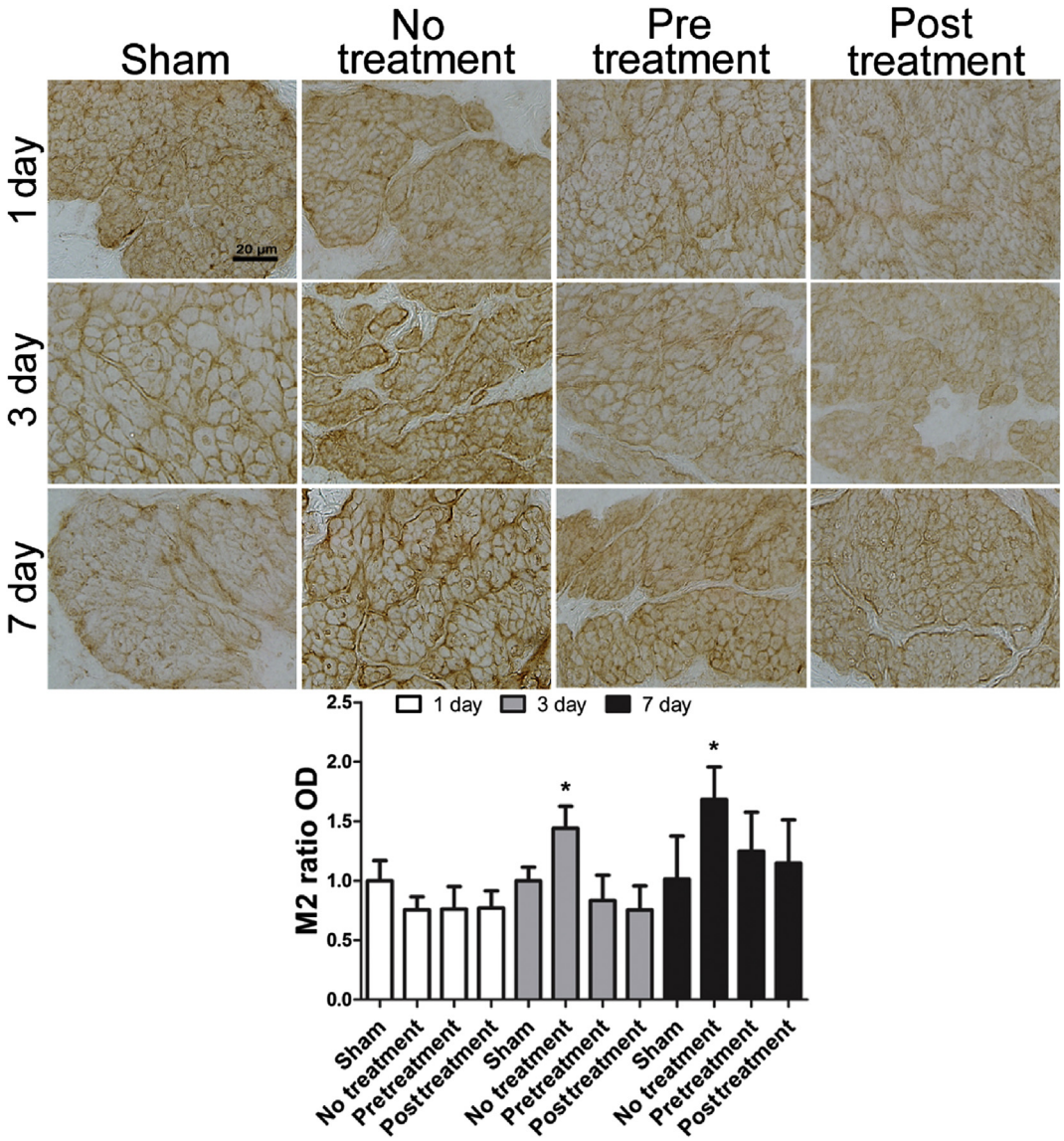

Figure 4. Temporal expressions of M_2 immunoreactivity in middle cerebral artery occlusion (MCAO) rat bladder (6 rats in each group). Expression of M_2 immunoreactivity increases at 3 days and 7 days after MCAO, but declines to the levels of the sham-operated group after $CD34^+$ treatment. Bar indicates 20 μm. *Compared with sham-operated group, $p < 0.05$. Pretreatment: preischemic treatment, posttreatment: postischemic treatment.

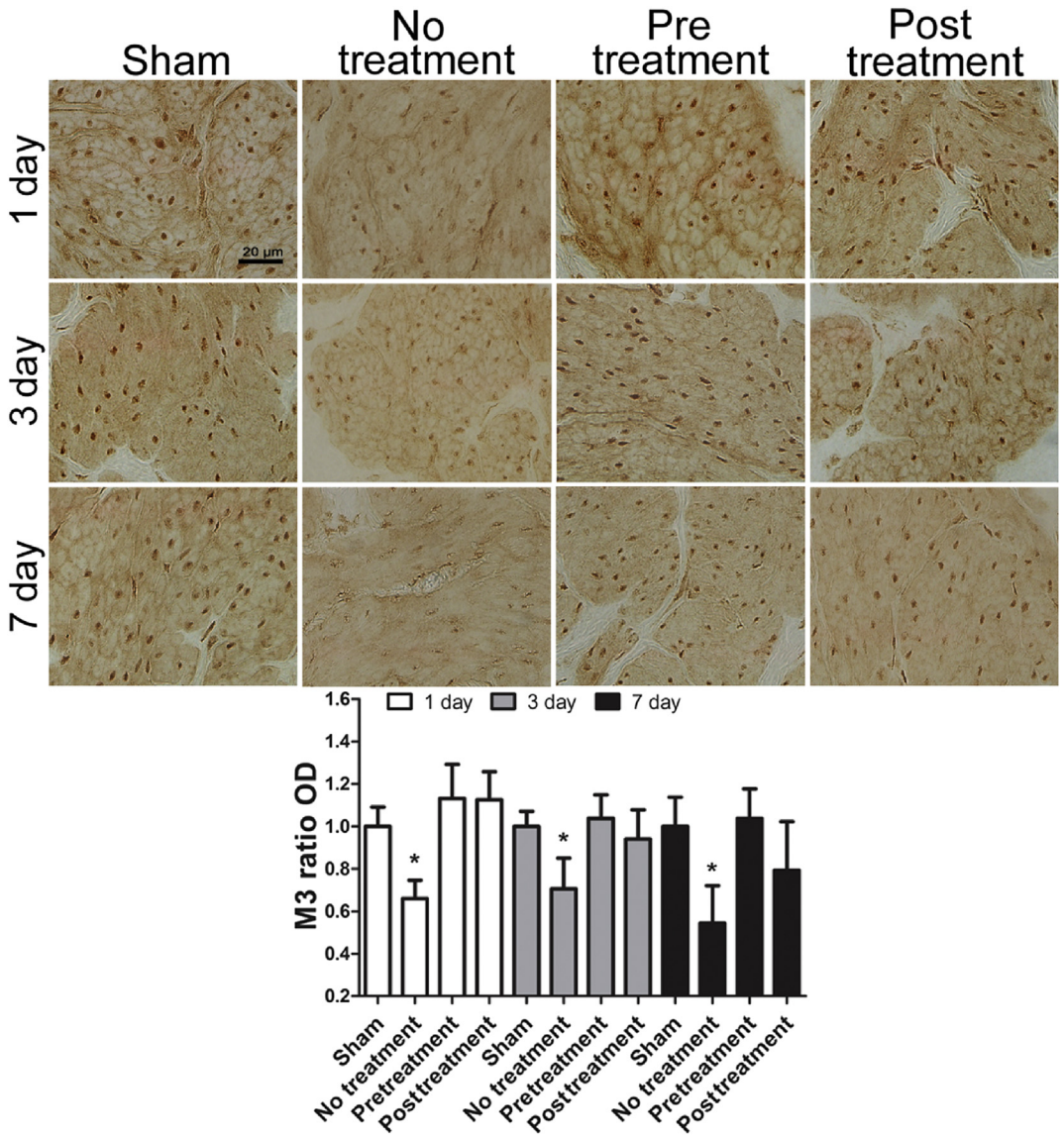

Figure 5. Temporal expressions of M_3 immunoreactivity in middle cerebral artery occlusion (MCAO) rat bladder (6 rats in each group). Expression of M_3 immunoreactivity significantly decreases at 1 day, 3 days, and 7 days after MCAO but increases after $CD34^+$ cell treatment. Bar indicates 20 μm. *Compared with sham-operated group, $p < 0.05$. Pretreatment: preischemic treatment, posttreatment: postischemic treatment.

preischemic $CD34^+$ cell treatment, postischemic $CD34^+$ cells transplantation did not upregulate the expression of NGF, M_2, and M_3 mRNA to the levels of the sham group at 7 days after MCAO.

Discussion

Voiding dysfunction is a common sequela of cerebral stroke [16]. Maruyama et al [9] reported that rats with cerebral infarction show a significant decrease in bladder capacity, resulting in a shortening of the micturition interval and a decrease in voided volume. The bladder capacity of cerebral-infarcted rats is significantly reduced which may be mediated by two mechanisms: (1) upregulation of an excitatory pathway from the forebrain; and (2) downregulation of a tonic inhibitory pathway from the forebrain [3]. Vendrame et al [17] measured infarct volume in an MCAO rat stroke model and demonstrated a dose relationship between HUCB cells, behavioral improvement, and neural sparing. However, no study has reported that HUCB cells can mediate therapeutic effects on bladder dysfunction after stroke. The cystometric data in the present study showed that rats undergoing MCAO have a significant increase in peak voiding pressure and residual volume. Conversely, voided volumes and intercontraction intervals significantly decrease after MCAO, but variables of urodynamic testing return to the levels of sham-operated rats after HUCB-derived $CD34^+$ cell treatment.

NGF is normally present in bladder muscle cells and urothelium [18]. Previous study showed that bilateral vesical artery ligation in rats may cause damage of detrusor muscle and there is decreased NGF immunofluorescence and elevated NGF mRNA in bladder [19]. Liang et al [14] found that NGF immunoreactivity and mRNA in the bladder muscle were transiently increased at 1 day, and declined significantly at 7 days and 28 days after bilateral common carotid artery occlusion in rats. The current results demonstrate that the decrease in bladder NGF immunoreactivity and mRNA expression that occurs in rats 3–7 days after MCAO can be reversed to an increase in NGF expression following preischemic $CD34^+$ cell treatment, due to an amelioration of the ischemic insult of brain. A

previous report indicates that cerebral stroke may modulate peripheral NGF levels [20]. Serum NGF levels were significantly related to stroke severity and the volume of brain infarction in the immediate poststroke period [20].

Rat bladder muscle is enriched with muscarinic receptors [21]. The mechanism underlying the regulation of muscarinic receptors in the bladder following cerebral infarction is still unclear. Some authors have suggested that cerebral infarction reduces parasympathetic tone in the bladder, thereby inducing compensatory upregulation of muscarinic receptors [9]. Results of our study show that expression of M_2 immunoreactivity in the bladder is increased, but M_3 immunoreactivity is decreased in the 7 days after MCAO. M_3 receptors are a minority population in this tissue, but they have been shown to play a predominant role in mediating most of the detrusor contractility under normal physiological conditions [22,23]. However, when compared with normal controls, there is a 50% increase in total receptor density and a 60% increase in M_2 but not M_3 receptor density in the denervated bladder [24]. Spinal cord injury in the rat may induce bladder hypertrophy and a change in muscarinic receptor subtype from M_3 toward M_2 [25]. Our data also reveal that the expression of M_2 mRNA and M_3 mRNA in the bladder decreases after MCAO in the untreated group. However, this expression may return to sham group levels after preischemic $CD34^+$ cell treatment.

In previous studies, systemic administration of HUCB-derived $CD34^+$ cells to MCAO rodents has been found to induce the neovascularization in the ischemic zone that is essential for neuronal regeneration and functional recovery after stroke [1]. Only a few studies regarding stem cell treatment in bladder diseases have been conducted [26–28]. Some investigators have reported that local injection of muscle-derived [28] or bone marrow stem cells may ameliorate impaired detrusor contractility in injured bladder rodent models [26,27]. However, adult stem cells from bone marrow and muscle are difficult to obtain, and they have the disadvantages of restricted differentiation potential, decreasing number with age, and a shorter life span compared with HUCB or amniotic fluid stem cells [29].

This study has some limitations. First, the present study did not demonstrate the therapeutic effect of $CD34^+$ cells on behavior recovery and on the infarct size in the ischemic brain. Further study is needed to illustrate the mechanism of neuroprotection by $CD34^+$ cells, which may promote hemodynamic and functional recovery of the brain after MCAO. Second, we did not use the menopausal rats instead of bilateral ovariectomized rats. Third, bladder injection of $CD34^+$ cells may improve bladder dysfunction induced by MCAO. Future research on this topic deserves to be conducted.

In conclusion, the present study shows that MCAO in rats results in changes of bladder NGF and muscarinic receptors and that such regulation of bladder receptors may be at least partly associated with the symptoms of bladder dysfunction induced by cerebral infarction. Our results demonstrate that bladder dysfunction in a rat model caused by MCAO may be restored to normal micturition by treatment with HUCB-derived $CD34^+$ cells and that this improvement may be related to the expression of NGF, M_2, and M_3 in the bladder.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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